

Vascular Actions of Brain Natriuretic Peptide: Modulation by Atherosclerosis and Neutral Endopeptidase Inhibition

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- OBJECTIVES** We sought to define the vascular actions of the cardiac hormone brain natriuretic peptide (BNP) on cellular proliferation and cyclic guanosine monophosphate (cGMP) in human aortic vascular smooth muscle cells (HAVSMCs). Secondly, we investigated BNP and acetylcholine (ACh) vasorelaxations in aortic rings from normal and atherosclerotic rabbits in the presence and absence of long-term oral inhibition of neutral endopeptidase (NEP).
- BACKGROUND** The vascular actions of BNP are not well defined, despite the presence of its receptor in vascular smooth muscle and the upregulation of NEP, the ectoenzyme that degrades BNP, in the vascular wall in atherosclerosis.
- METHODS** HAVSMCs stimulated with fetal calf serum (FCS) were pulsed with bromodeoxyuridine (BrdU) with and without BNP. The HAVSMCs were incubated in the presence and absence of BNP to assess cGMP. Vasorelaxations to BNP and ACh were assessed in rings in normal and atherosclerotic rabbits in the presence and absence of long-term oral inhibition of NEP, together with assessment of atheroma formation.
- RESULTS** FCS-stimulated BrdU uptake in HAVSMCs was suppressed with BNP. BNP potentiated cGMP in HAVSMCs. BNP resulted in potent vasorelaxation in normal isolated aortic rings, which were impaired in atherosclerotic versus normal rabbits and preserved with NEP inhibition, which also decreased atheroma formation. Relaxations to ACh, which were also impaired in atherosclerosis, were preserved with inhibition of NEP.
- CONCLUSIONS** We conclude that BNP potently inhibits vascular smooth muscle cell proliferation and potentiates the generation of cGMP. BNP potently relaxes the normal rabbit aorta, and this response is impaired in atherosclerosis but preserved with inhibition of NEP, together with a reduction in atheroma formation and preservation of relaxations to ACh. (J Am Coll Cardiol 2000;35:796–801) © 2000 by the American College of Cardiology

Brain natriuretic peptide (BNP) is a member of a family of structurally similar but genetically distinct peptides that includes atrial and C-type natriuretic peptides (1–3). Brain natriuretic peptide has been viewed as a volume-regulating hormone of cardiac origin that mediates renal and endocrine actions through activation of particulate guanylyl cyclase

(PGC) receptors and the generation of cyclic guanosine monophosphate (cGMP) (4–6).

Although BNP functions as a circulating cardiac hormone in the control of body fluid homeostasis (7), the natriuretic peptide A receptor (NPR-A) that binds BNP is also present in the vascular wall, underscoring a potential role for BNP in the control of vascular function through cGMP (8). Thus, BNP may function in parallel with nitric oxide (NO), which is also a potent activator of cGMP through activation of soluble guanylyl cyclase (9). The biologic importance of BNP in vascular regulation is supported by recent studies that have reported that acute natriuretic peptide receptor blockade *in vivo* increases coronary vascular resistance (10), whereas genetic disruption of the NPR-A receptor results in a model of hypertension, left ventricular hypertrophy and sudden death (11). Studies have also demonstrated that the biologic actions of the natriuretic peptides are limited through rapid enzymatic degradation by

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Manuscript received September 2, 1998; revised manuscript received September 13, 1999, accepted November 15, 1999.

Abbreviations and Acronyms

ACh	= acetylcholine
BNP	= brain natriuretic peptide
BrdU	= bromodeoxyuridine
cGMP	= cyclic guanosine monophosphate
EC ₅₀	= effective concentration at 50% relaxation
FCS	= fetal calf serum
HAVSMC	= human aortic vascular smooth muscle cell
NEP	= neutral endopeptidase
NO	= nitric oxide
NPR-A	= natriuretic peptide A receptor
PGC	= particulate guanylyl cyclase

the membrane-bound ectoenzyme neutral endopeptidase (NEP), which has recently been shown to be increased in the vascular wall in experimental atherosclerosis (12,13). Indeed, long-term NEP inhibition has been reported to decrease atheroma formation and preserve endothelium-dependent vasorelaxations in association with a decrease in serum cholesterol (13).

The current studies were designed to better define the vascular biology of BNP and to confirm the role of chronic NEP inhibition in modulating atheroma formation and preserving endothelium-dependent vasorelaxations in experimental atherosclerosis. Specifically, we first determined the ability of BNP to inhibit stimulated cell proliferation in cultured human aortic vascular smooth muscle cells (HAVSMCs) and to augment cGMP generation. Secondly, we defined the ability of BNP to relax precontracted isolated aortic rings from normal rabbits in the presence and absence of the endothelium. Thirdly, we determined whether the vasorelaxing actions to BNP are impaired in atherosclerosis and whether chronic NEP inhibition would preserve vasorelaxations to BNP as well as endothelium-dependent relaxations in association with a reduction in atheroma formation.

We hypothesized that BNP would inhibit stimulated cell proliferation and augment cGMP generation in HAVSMCs. In addition, we hypothesized that BNP is a potent endothelium-independent vasorelaxing factor in isolated rabbit aortic rings and that this effect is attenuated in a model of experimental atherosclerosis produced by hypercholesterolemia but preserved by chronic oral inhibition of NEP. Such restoration of BNP relaxations by NEP inhibition would be associated with a decrease in atheroma formation and a preservation of endothelium-dependent relaxations to acetylcholine (ACh), independent of decreases in serum cholesterol.

METHODS

Studies in cultured HAVSMCs. Cell proliferation studies were performed according to previously described methods (14). The HAVSMCs (passages 4 through 6) (Clonetics Corporation Inc., San Diego, California) were grown to

confluence in culture media supplemented with required growth factors, passaged in 96 well plates and incubated with supplemented smooth muscle cell media for 24 h. The supplemented medium was replaced with basal smooth muscle cell media for 24 h to render the cells quiescent. The cells were subsequently incubated in the presence or absence of serum-supplemented media (5%), and in the presence of serum-supplemented growth media along with exogenous human BNP (10^{-7} mol/liter) for 24 h. Proliferation was assessed with bromodeoxyuridine (BrdU) uptake over the next 24 h, as previously described (14).

Separate HAVSMCs (passages 4 through 6) were grown to confluence in smooth muscle cell basal media supplemented with the required growth factors. The cells were passaged to 24 well culture dishes and incubated in supplemented media and again grown to confluence over 48 h. The supplemented medium was replaced with basal media to render the cells quiescent for 24 h before incubation with experimental conditions. The cells were then incubated in the presence or absence of BNP (10^{-7} mol/liter) and 3-isobutyl-1-methyl xanthine, a phosphodiesterase inhibitor, for the indicated periods. Media were collected from the wells, frozen and stored at -20°C until radioimmunoassay was performed to measure cGMP levels by methods previously described (15).

Studies of vascular reactivity and atheroma formation.

Male New Zealand white rabbits were used in these studies. The rabbits were housed individually in stainless-steel, wire-bottomed cages in a room controlled with a 12-h light–dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Rabbits weighing 3.6 ± 0.1 kg were assigned to three groups: normal ($n = 8$), cholesterol fed ($n = 7$) and cholesterol fed and treated with an orally active NEP inhibitor, Candoxatril (UK 79300, Pfizer, United Kingdom) ($n = 6$). The normal group was fed a normal diet (150 g/day) for eight weeks. The cholesterol-fed group received a 1% cholesterol diet (150 g/day) for eight weeks to produce atherosclerosis, as previously reported (16). The NEP inhibition group received a 1% cholesterol diet (150 g/day) for eight weeks and Candoxatril (20 mg/kg body weight per day) dissolved in drinking water. After eight weeks the animals were sacrificed with an overdose of intravenous pentobarbital, and the aortas were excised and cleaned of connective tissue on a towel soaked in cold Krebs solution. The aortas were sectioned into 3- to 5-mm rings. Some rings were denuded of endothelium by inserting a pair of fine forceps into the lumen and gently rolling the ring back and forth on Krebs-Ringer-wetted paper. Rings with and without endothelium from individual rabbits were studied in parallel.

After harvesting and cleaning the vessels, the aortic rings

were suspended in organ chambers filled with aerated (95% oxygen and 5% carbon dioxide) modified Krebs-Ringer bicarbonate solution (composition in mmol/liter: 118.3 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 25.0 NaHCO₃, 0.026 calcium sodium EDTA and 11.1 dextrose-control solution) at 37°C. During a 1- to 2-h equilibration period, the maximal contractile response to KCl was determined at previously determined optimal points in the length-tension relation. The rings were then precontracted with phenylephrine (10^{-7.5} to 10^{-8.5} mol/liter) until stable contraction was attained. The rings were then exposed to cumulative increases in concentration of ACh and BNP in half-log increments (10⁻¹¹ to 10^{-8.5} mol/liter).

In assessment of atheroma formation, the thoracic aortas up to the origin of the second intercostal artery were used to quantify plaque by oil-red O staining in rabbits fed a 1% cholesterol diet for 10 weeks (n = 6) and rabbits fed a 1% cholesterol diet while receiving Candoxatril (20 mg/kg per day) dissolved in drinking water (n = 8). After immersion fixation overnight in 10% neutral buffered Formalin, the thoracic aorta was stained with oil-red O (0.3%). A single longitudinal incision along the wall opposite the arterial ostia was made, and the vessels were pinned open and photographed. The percent plaque area was determined from the values for the total area examined and the stained area by threshold analysis using true-color image analyzer software.

Statistical analysis. All data are expressed as the mean value ± SEM. Statistical evaluation of data was performed using the Student *t* test for unpaired observations. When more than two groups were compared, analysis of variance with the Bonferroni correction was used. Values of *p* < 0.05 were considered significant. All statistical analysis was performed on GraphPad Prism (version 2.0a) software.

RESULTS

Studies in cultured HAVSMCs. Figure 1A reports BNP-mediated inhibition of fetal calf serum (FCS)-stimulated replication in HAVSMCs as assessed by BrdU uptake. The FCS increased BrdU uptake in HAVSMCs as compared with cells incubated with smooth muscle cell basal media (5% FCS 2.2 ± 0.1 optical density units vs. smooth muscle cell basal media 0.58 ± 0.07; n = 6, *p* < 0.05). Coincubation of exogenous BNP with FCS resulted in a decrease in cell proliferation (FCS 2.2 ± 0.1 vs. FCS + BNP 0.9 ± 0.29; *p* < 0.05). Figure 1B reports cGMP generation and BNP in HAVSMCs, demonstrating that BNP stimulated cGMP accumulation at 3 h (1.13 ± 0.19 pmol/ml vs. undetectable; *p* < 0.05), 9 h (1.63 ± 0.13 pmol/ml vs. undetectable; *p* < 0.05) and 30 h (3.78 ± 0.24 vs. 1.43 ± 0.23 pmol/ml; *p* < 0.05).

Studies of vascular reactivity and atheroma formation. Table 1 reports serum lipids and mean arterial pressure in the normal, cholesterol-fed and cholesterol-fed plus NEP

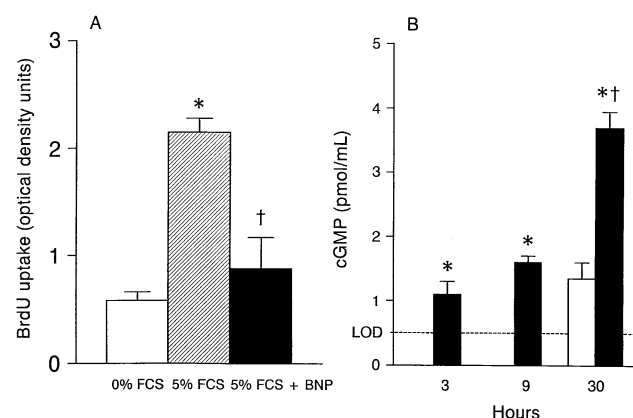


Figure 1. Left, Bar graph showing BrdU uptake in optical density units as measured by colorimetry. **p* < 0.05 vs. 0% FCS. †*p* < 0.05 vs. 5% FCS. 0% FCS = BrdU uptake in cells not stimulated with FCS; 5% FCS = BrdU uptake in cells stimulated with 5% FCS; 5% FCS + BNP = BrdU uptake in cells coincubated in 5% FCS and BNP (10⁻⁷ mol/liter). Right, Bar graph showing cGMP generation. **p* < 0.05 vs. control. †*p* < 0.05 vs. 3 and 9 h. LOD = limit of detection.

inhibition groups. There was no difference between the groups in mean arterial pressure. Total cholesterol, triglycerides and low density lipoprotein cholesterol were markedly elevated in the cholesterol-fed group in the absence of changes in high density lipoprotein cholesterol. In the group with long-term NEP inhibition, serum lipids were not altered, as compared with the cholesterol-fed group.

Figure 2A reports dose-dependent vasorelaxations to BNP with and without the endothelium. BNP induced potent relaxations (100% maximal relaxation) in normal isolated aortic rings precontracted with phenylephrine. Relaxations were unaffected by removal of the endothelium. Figure 2B reports relaxations to BNP in normal, atherosclerotic and atherosclerotic plus NEP inhibition groups, with

Table 1. Hemodynamic Data and Serum Lipids

Measurements*	Normal†	Cholesterol‡	NEPI§
MAP (mm Hg)	86 ± 2	80 ± 1.88	82.5 ± 1.6
Total cholesterol (mg/dl)	39 ± 3	1,725 ± 119	1,720 ± 427
Total triglycerides (mg/dl)	118 ± 16	336 ± 77	265 ± 54
HDL cholesterol (mg/dl)	27 ± 5	23 ± 3	26 ± 7
LDL cholesterol (mg/dl)	3.60 ± 1.17	1,579 ± 126	1,514 ± 367

*Measurements were obtained before death, eight weeks after initiation of the diet. †Normal indicates rabbits fed standard chow. ‡Cholesterol indicates rabbits fed a 1% cholesterol diet. §NEPI indicates rabbits fed a 1% cholesterol diet and 20 mg/kg per day of Candoxatril, an orally active NEP inhibitor. ||*p* < 0.05 versus normal. Data are presented as the mean value ± SEM.

HDL = high density lipoprotein; LDL = low density lipoprotein; MAP = mean arterial pressure; NEP = neutral endopeptidase; NEPI = neutral endopeptidase inhibitor (Candoxatril).

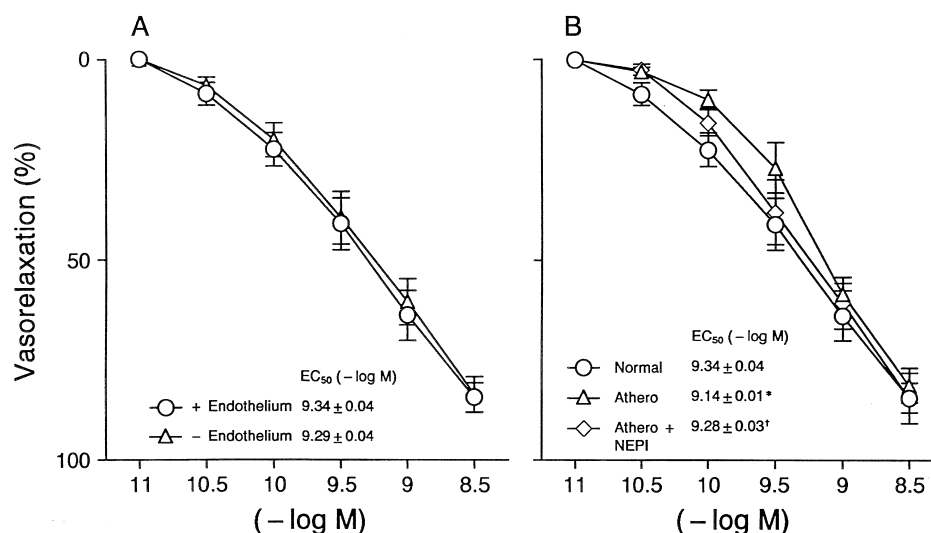


Figure 2. Left, Relaxation curves to BNP in the presence (open circles) and absence (open triangles) of endothelium in precontracted aortic rings from normal rabbits. Right, Relaxation curves to BNP in the presence of endothelium in precontracted aortic rings from normal rabbits (open circles), atherosclerotic rabbits (open triangles) and atherosclerotic rabbits treated for eight weeks with the orally active NEP inhibitor (NEPI), Candoxatril (UK 79300) (open diamonds). **p* < 0.05 vs. normal. †*p* < 0.05 vs. atherosclerosis.

their associated effective concentration at 50% relaxation (EC₅₀). The BNP-induced relaxations were impaired in atherosclerosis in association with a decrease in EC₅₀. Long-term NEP inhibition in cholesterol-fed rabbits preserved relaxations to BNP. Relaxations to ACh were also impaired in atherosclerosis (EC₅₀, 7.10 ± 0.03* vs. 7.42 ± 0.06, -log mol/liter, **p* < 0.05) and preserved with NEP inhibition (EC₅₀, 7.65 ± 0.03, -log mol/liter).

Figure 3 reports atherosclerotic plaque area as quantified by oil-red O staining. Cholesterol-fed rabbits showed significant atheroma formation in the thoracic aorta (48 ± 5%), which was significantly (*p* < 0.05) decreased with NEP inhibition (32 ± 3%) in the absence of decreases in serum cholesterol (Table 1).

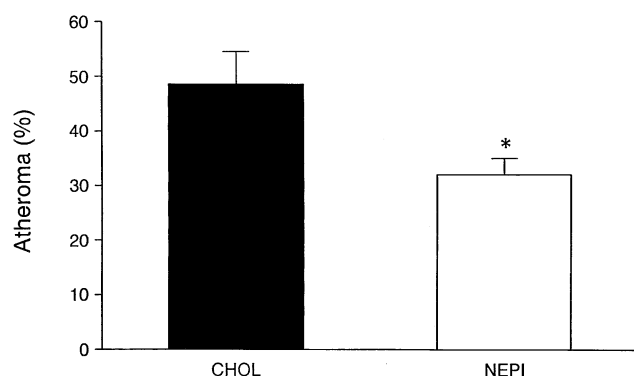


Figure 3. Bar graph showing atherosclerotic plaque area. CHOL = cholesterol-fed untreated rabbits (*n* = 6). NEPI = cholesterol-fed rabbits treated with Candoxatril (UK 79300) (*n* = 8). **p* < 0.05 vs. cholesterol.

DISCUSSION

The current studies were designed to address the vascular biology of BNP. We report that BNP potently inhibits proliferation in cultured HAVSMCs stimulated by FCS and also significantly augments the generation of cGMP. In addition, we report that BNP potently relaxes isolated rabbit aortic rings independent of the endothelium, and that this action is attenuated in experimental atherosclerosis. Importantly, these investigations also report that long-term oral inhibition of NEP, the ectoenzyme that degrades the natriuretic peptides, preserves relaxations to BNP in experimental atherosclerosis in association with a reduction in atheroma formation and preservation of endothelium-dependent relaxations to ACh, independent of decreases in elevated serum cholesterol.

Antiproliferative actions of BNP. Previous reports have demonstrated that atrial and C-type natriuretic peptides possess antimitogenic actions in vascular smooth muscle cells through the NPR-A and NPR-B receptors, respectively, which are both linked to particulate guanylate cyclase and the generation of cGMP (17-21). To date, the effects of BNP on vascular smooth muscle replication have been incompletely defined. The importance of focusing on the vascular biology of BNP is underscored by reports that BNP may be the most biologically potent of the natriuretic peptides with regard to its renal actions and its gene activation in response to growth factors (22,23). We report, for the first time to our knowledge, that BNP has antireplicative actions in cultured HAVSMCs. This observation complements recent studies that have demonstrated that

BNP inhibits oxidized low density lipoprotein-induced migration of cultured HAVSMCs (24).

Cyclic cGMP actions of BNP. The current studies also demonstrate, for the first time to our knowledge, that BNP augments cGMP generation in HAVSMCs. Thus, BNP may function as a vasoactive factor, similar to NO, which also activates cGMP in vascular smooth muscle cells. Therefore, BNP and NO represent a humoral pathway that has antiproliferative and vasorelaxing actions secondary to cGMP generation through activation of particulate and soluble guanylyl cyclase, respectively (1). Further studies are needed to further characterize the potential interactions between these two pathways, one of which is primarily related to circulating factors (i.e., BNP) and the other which is primarily local (i.e., NO).

Vasorelaxing actions of BNP. Although vasorelaxations to atrial and C-type natriuretic peptides have been well defined, less is known about vasorelaxations to BNP (25). In addition, although vasorelaxations to atrial and C-type natriuretic peptides in experimental atherosclerosis have recently been reported to be attenuated (13), the modulating effect of atherosclerosis on vasorelaxations to BNP has not been reported. The current study therefore importantly extends previous investigations. First, we report that BNP potently and completely relaxes the isolated rabbit aorta, and this action is not dependent on the endothelium. Further, these vasorelaxing actions are attenuated at low concentrations in the setting of experimental atherosclerosis produced by hypercholesterolemia. Thus, experimental atherosclerosis emerges as a disease state in which the vasorelaxing actions of all the natriuretic peptides (BNP and atrial and C-type natriuretic peptides) are impaired in association with attenuation of the NO pathway—two systems linked to cGMP.

Antiatherogenic actions of NEP inhibition. The ectoenzyme NEP is a widely distributed enzyme that rapidly degrades the natriuretic peptides, thus limiting their biologic actions. One report (13) documents that vascular wall NEP is increased in the model of atherosclerosis we used in this study. Such enhanced NEP activity could account in part for the reduced vascular responsiveness to BNP, as well as to atrial and C-type natriuretic peptides, as reported in this previous investigation (13). Consistent with such an interpretation is the additional report that inhibition of NEP preserves vasorelaxation to atrial and C-type natriuretic peptides (13). We report in the current study of atherosclerosis induced by a high cholesterol diet that long-term oral inhibition of NEP preserves vasorelaxations to BNP and attenuates atheroma formation in association with preservation of endothelium-dependent relaxations to ACh.

The current investigation is in contrast to previous reports in which the antiatherogenic effect of NEP inhibition occurred in association with a decrease in serum cholesterol (13). As we observed no decrease in serum cholesterol, the current observation suggests that the decrease in atheroma formation to NEP inhibition is not due to cholesterol lowering. Our finding that BNP has antimitogenic effects in cultured HAVSMCs

suggests that BNP could play a role in inhibiting vascular smooth muscle cell proliferation in vivo. In addition, BNP inhibits the migration of human coronary vascular smooth muscle cells in vitro, and thus may play a role in preventing migration in vivo (24). Consequently, augmentation of BNP through NEP inhibition could account in part for the decreased atheroma formation we report in the current study. In addition, augmentation of BNP through NEP inhibition may also stimulate local release of C-type natriuretic peptide, as reported by Nazario *et al.* (26), which could further inhibit vascular smooth muscle cell replication and migration. The antiatherogenic effect we report with long-term NEP inhibition is also not due to decreases in blood pressure, as there was no difference in blood pressure between the cholesterol-fed and the cholesterol-fed plus NEP inhibition groups.

Strategies for attenuating the pathogenesis of atherosclerosis and its characteristic vascular wall dysfunction include inhibition of deleterious growth-promoting and vasoconstricting factors as well as augmentation of antiproliferative and vasorelaxing factors. Specifically, angiotensin-converting enzyme inhibition and L-arginine have been attractive experimental therapeutic strategies that can be combined with lipid-lowering agents (27–30). Such approaches have resulted in preservation of endothelium-dependent vasorelaxations in the setting of atherosclerosis through enhancement of endothelium-derived NO production and other undefined mechanisms. Indeed, a recent study reported the preservation of endothelium-dependent relaxations to ACh in the setting of atherosclerosis with long-term oral inhibition of NEP (13). To our knowledge, our results are the first to confirm this important finding and support the use of another pharmacologic agent, which through cGMP secondary to activation of the natriuretic peptides, preserves endothelium-dependent and -independent function.

Conclusions. We report, for the first time to our knowledge, that BNP has antireplicative effects on cultured HAVSMCs in association with activation of cGMP generation. In addition, we report that vasorelaxations to BNP in the normal rabbit aorta are endothelium independent and impaired in experimental atherosclerosis. Finally, our results demonstrate that long-term oral inhibition of NEP preserves relaxations to BNP in atherosclerosis, while attenuating atheroma formation and maintaining endothelial function independent of reductions in serum cholesterol.

Acknowledgments

The authors gratefully acknowledge the technical assistance of Sharon Sandberg, Gail Harty and Denise Heublein and the secretarial help of Linda Combs.

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